APOPTOSIS AND GENOTOXICITY OF SELECTED β-CARBOLINE COMPOUNDS AGAINST CHRONIC MYELOGENOUS LEUKEMIA USING *IN VITRO* EXPERIMENTAL MODELS

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by

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treated negative control counterparts. The results shown are the mean ± SEM from at least three independent experiments, whereby each was conducted in triplicate. For statistical analysis, one-way ANOVA was conducted, which followed by Bonferroni's multiple comparison post test. Significance is denoted as ***$P < 0.001$.

LIST OF SYMBOLS AND ABBREVIATIONS

β	Beta
°C	Degree Celsius
+	Positive or plus
-	Negative or minus
×	Times or multiply
/	Per or divide
±	Plus and minus
<	Less than
>	More than
≤	Equal to or less than
≥	Equal to or more than
~	Approximately
%	Percentage
=	Equal to
2-AA	2-aminoanthracene
2N	Diploid
2NF	2-nitrofluorene
4N	Tetraploid
4NQO	4-nitroquinoline N-oxide
5-FU	5-fluorouracil
A	Apoptosis
ABC	ATP-binding-cassette
ADME	Absorption, distribution, metabolism and elimination
AIF	Apoptosis inducing factor
ALL	Acute lymphocytic (or lymphoblastic) leukemia
AML	Acute myeloid (or myelogenous) leukemia
ANOVA	One-way analysis of variance
AO	Acridine orange
APAF1	Apoptotic protease-activating factor 1
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
B	Binomial significance value
BALB/c3T3	BALB/3T3 clone A3, mouse embryonic fibroblast cell line (ATCC No. CCL-163)
BCL-2	B-cell lymphoma 2
BCRP	Breast cancer resistance protein
BP	Benzo(a)pyrene
Br	Bromine

C	Carbon
CBS	Calf bovine serum
CDK	Cyclin-dependent kinase
cells/mL	Cells per milliliter
CEN	Chicken erythrocyte nuclei
CH_3	Methyl
CHAT	Deoxycytidine, hypoxanthine, aminopterin and thymidine
CHO	Chinese hamster ovary
CHT	Deoxycytidine, hypoxanthine and thymidine
CLL	Chronic lymphocytic leukemia
cm	Centimetre
cm^2	Square centimetres
cm^3	Cubic centimetres
CML	Chronic myeloid (or myelogenous) leukemia
Cn	Optical density for vehicle control
CO_2	Carbon dioxide
COM	UK Committee on Mutagenicity of Chemicals
COOH	Carboxyl
CTA	Cell transformation assay
CTN	Calf thymocyte nuclei
DMEM	Dulbecco's modified Eagle's medium
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DYRK1A	Dual enzyme tyrosine phosphorylated and regulated kinase 1a
EB	Ethidium bromide
EMEA	European Medicines Agency
EMEM	Eagle's Minimum Essential Medium
ESI	Electrospray ionization
EU	European Union
EURL ECVAM	The EU Reference Laboratory for Alternatives to Animal Testing
EW	Empty wells
F	Fluorine
FADD	FAS-associated death domain
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FT-IR	Fourier transform infrared
G_0 phase	Gap 0 phase
G_1 phase	Gap 1 phase

G_2 phase	Gap 2 phase
G6P	Glucose-6-phosphate
g/mol	Gram per mol
H	Hydrogen
H_2O_2	Hydrogen peroxide
HeLa	Human cervix adenocarcinoma cell line (ATCC No. CCL-2)
Hep G2	Human hepatocellular carcinoma cell line (ATCC No. HB-8065)
his	Histidine
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
HRR	Homologous recombination repair
Hs27	Human foreskin fibroblast (ATCC No. CRL-1634)
HT-29	Human colorectal adenocarcinoma (ATCC No. HTB-38)
IARC	International Agency for Research on Cancer
IC_{50}	Half maximal (50%) inhibitory concentration
IC_{100}	Maximal (100%) inhibitory concentration
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
ICEATM	Interagency Center for the Evaluation of Alternative Toxicological Methods
IκK	IκB kinase complex
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
IMDM	Iscove's Modified Dulbecco's Medium
K562	Human chronic myelogenous leukemia cell line (ATCC No. CCL-243)
KCl	Potassium chloride
LC50	Lethal concentration at 50% viability
LPS	Lipopolysaccharide
M	Molar
MDR	Multidrug resistance
MF	Mutation frequency
Mg	Magnesium
mg/kg	Milligram per kilogram
mg/mL	Milligram per millilitres
MK-2	Mitogen activated protein kinase-activated protein kinase 2
mL	Millilitres
MLA	Mouse lymphoma assay
mM	Millimolar
mm	Millimetres

mm^2	Square millimetres
mm^3	Cubic millimetres
mmol	Millimole
MN	Micronucleus
MNi	Micronuclei
MPF	Microplate format
M phase	Mitotic phase
MRP1	Multidrug resistance-associated protein 1
MS	Mass spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	Necrosis
N	Nitrogen
NADH	Nicotinamide adenine dinucleotide hydrate
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate hydrate
NaH	Sodium hydride
NCI	National Cancer Institute
ND	Not determined
NF	Nitrofluorene
NG	Normal growing
NHEJ	Non-homologous end-joining
nm	Nanometre
NMR	Nuclear magnetic resonance
NTP	National Toxicology Program
O	Oxygen
OECD	Organisation for Economic Co-operation and Development
OD	Optical density
P	Probable number of colonies per well
$*P$	Significant difference at P-value of less than 0.05
$**P$	Significant difference at P-value of less than 0.01
$***P$	Significant difference at P-value of less than 0.001
PARP-1	Poly (ADP-ribose) polymerase-1
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PE	Plating efficiency
Pgp	P-glycoprotein
PLK1	Polo-like kinase 1
PI	Propidium iodide
RCC	Relative cell count
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals

RFU	Relative Fluorescence Unit
RICC	Relative increase in cell counts
RLU	Relative Luminescence Unit
RNA	Ribonucleic acid
RNase	Ribonuclease
RO5	Lipinski's rule of five
RPD	Relative population doubling
rpm	Revolutions per minute
RS	Relative survival
S	Sulphur
S9	Post mitochondrial fraction prepared from the livers of rodents
SAR	Structure activity relationship
SD	Standard deviation
SEM	Standard error of the mean
SG	Slow growing
SHE	Syrian hamster embryo
SI	Selectivity index
SMAC	Second mitochondria-derived activator of caspases
S. typhimurium	*Salmonella typhimurium*
T_0	Optical density at time zero
tAIF	Truncated apoptosis inducing factor
tBID	Truncated BH3 interacting-domain death agonist
TFT	Trifluorothymidine
TGI	Total growth inhibition
TK gene	Thymidine kinase gene
TK6	Human lymphoblast cell line (ATCC No. CRL-8015)
TKI	Tyrosine kinase inhibitor
TLC	Thin-layer chromatography
TNF	Tumour necrosis factor
TNFR1	Tumour necrosis factor receptor 1 protein
TRADD	TNFR1-associated death domain protein
Trypsin-EDTA	0.25% Trypsin - 0.02% EDTA
TW	Total wells
µg/mL	Microgram per millilitre
µL	Microlitre
µM	Micromolar
V	Viable
v/v	Volume per volume
WHO	World Health Organization
w/v	Weight per volume
XIAP	X-linked inhibitor of apoptosis protein

APOPTOSIS DAN GENOTOKSIK BAGI SEBATIAN β-KARBOLINA TERHADAP LEUKEMIA MIELOGENUS KRONIK DENGAN MENGGUNAKAN MODEL EKSPERIMEN *IN VITRO*

ABSTRAK

β-karbolina dikenali dengan banyak kegunaan tradisional, dan telah diselidik secara meluas untuk pelbagai indikasi kesihatan. Khususnya, baru-baru ini β-karbolina telah menunjukkan beberapa penemuan yang memberangsangkan dalam bidang antikanser. Maka kajian ini menyelidiki potensi sebatian β-karbolina bagi aktiviti antikanser menggunakan kaedah model *in vitro.* Tiga puluh sebatian β-karbolina yang disintesis telah diuji menggunakan asai 3-(4,5-Dimetilthiazol-2-yl)-2,5-difeniltetrazolium bromida (MTT) untuk aktiviti antikanser dan indeks selektiviti (SI) terhadap empat jenis sel kanser manusia (kolon HT-29, serviks HeLa, hati Hep G2 dan leukemia K562) dan dua jenis sel bukan kanser (fibroblas embrio tikus BALB/c3T3 dan fibroblas kulit khatan manusia Hs27). Berdasarkan selektiviti yang tertinggi, tujuh sebatian telah dipilih untuk sel leukemia mielogenus kronik (CML) K562. Beberapa eksperimen telah dilakukan untuk memahami ciri-ciri sebatian tersebut dan menyelidik mekanisme tindakan separa bagi sel K562. Keputusan daripada asai MTT mencadangkan sebatian yang terpilih boleh menghasilkan aktiviti menghalang pertumbuhan sel secara maksima (IC_{100}) dari kepekatan 9.1 μM hingga 56.9 μM dalam 48 jam. Selain itu, sebatian tersebut bertindak sebagai agen sitostatik dan sitosidal apabila digunakan masing-masing pada kepekatan yang rendah dan tinggi. Kajian mekanisme tindakan separa menerusi pewarnaan flouresens akridina jingga dan etidium

bromida (AO/EB) dan asai luminesens kaspase-3 dan -7 telah menunjukkan yang sebatian terpilih mencetus apoptosis terhadap sel K562 tanpa peningkatan tahap kaspase, maka boleh menyimpulkan bahawa apoptosis bukan klasik bebas kaspase adalah bertanggungjawab. Sebagai tambahan, analisis kitaran sel menerusi aliran sitometri menunjukkan gangguan pada peringkat awal disebabkan pemberhentian pertumbuhan sel pada fasa G_0/G_1. Kajian keselamatan dalam bentuk genotoksik dikaji dengan selanjutnya menggunakan beberapa ujian genotoksik dengan dan tanpa kehadiran fraksi S9 dari hati tikus untuk pengaktifan metabolism. Ujian Ames bakteria *Salmonella typhimurium* dan ujian gen timidina kinase (*TK*) sel mamalia telah dijalankan untuk menyelidiki mutagenisiti, manakala ujian mikronukleus (MN) dan asai sel transformasi (CTA) yang bebas tambatan telah dijalankan untuk menyelidiki genotoksik dan karsinogenik masing-masing. Eksperimen-eksperimen *in vitro* ini telah membuktikan yang sebatian tersebut tidak genotoksik, tidak mutagenik dan tidak karsinogenik. Maka boleh dicadangkan bahawa ketujuh-tujuh sebatian β-karbolina telah menunjukkan potensi yang baik untuk dikembangkan pada masa hadapan sebagai agen antikanser, khususnya terhadap CML, berdasarkan potensi, selektiviti dan keselamatan *in vitro*.

APOPTOSIS AND GENOTOXICITY OF SELECTED β-CARBOLINE COMPOUNDS AGAINST CHRONIC MYELOGENOUS LEUKEMIA USING *IN VITRO* EXPERIMENTAL MODELS

ABSTRACT

β-carboline is known for its numerous traditional uses and has been widely investigated for various health indications. In particular, recently β-carboline has shown some promising findings in the field of anticancer. Hence this study investigates the potential anticancer activity of β-carboline compounds using *in vitro* models. Thirty synthesized β-carboline compounds were tested using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for anticancer activity and selectivity index (SI) against four human cancer (colon HT-29, cervical HeLa, liver Hep G2 and leukemia K562) and two non-cancer (mouse embryo fibroblasts BALB/c3T3 and human foreskin fibroblasts Hs27) cell lines. Based on the highest selectivity, seven compounds were selected for K562 chronic myelogenous leukemia (CML) cells. A series of experiments were further performed to understand the characteristics of the compounds as well as to investigate their partial mechanism of action in K562 cells. Results from MTT assay have indicated that the selected compounds were able to induce maximal inhibitory activity (IC_{100}) at a range of concentrations from 9.1 µM to 56.9 µM at 48 hours. Furthermore, the compounds acted both as cytostatic and cytocidal agents, when treated at low and high concentrations respectively. Partial mechanism of action studies by acridine orange and ethidium bromide (AO/EB) fluorescence staining and caspase-3 and -7 luminescence assay have shown that the selected compounds induced apoptosis in

K562 cells with no upregulation of the caspases, and therefore it could be deduced that non-classical caspase-independent apoptosis was responsible. In addition, cell cycle analysis by flow cytometry showed disruption at the initial stage due to cell arrest at G_0/G_1 phases. Safety studies in the form of genotoxicity were further evaluated using a battery of genotoxicity tests in the presence and in the absence of rat liver S9 metabolic activation. *Salmonella typhimurium* bacteria Ames test and mammalian cell thymidine kinase (*TK*) gene test were conducted for mutagenicity investigation, while micronucleus (MN) test and anchorage-independent cell transformation assay (CTA) were performed for genotoxicity and carcinogenicity investigations respectively. These *in vitro* experiments have proven that the compounds were non-genotoxic, non-mutagenic and non-carcinogenic. Hence, it could be postulated that these seven compounds of β-carboline have shown good potential to be developed in the future as antileukemic agents, specifically against CML, due to their *in vitro* potency, selectivity and safety.

CHAPTER 1

1.0 INTRODUCTION

1.1 Anticancer

1.1.1 Definition of cancer

Cancer is characterized by abnormal, excessive, uncoordinated, and autonomous proliferation of cells, even after the cessation of stimulus for growth which caused it (Yadav and Nandi, 2014). Cancer is thought to result from the accumulation of multiple genetic aberrations that transform normal cells into cancer cells (Cheon and Orsulic, 2011).

1.1.2 Cancer statistics

Cancer is now one of the leading causes of morbidity and mortality worldwide. World Health Organization (WHO), through its cancer research agency, International Agency for Research on Cancer (IARC) has conducted GLOBOCAN project, which aimed to provide contemporary estimates of the incidence of mortality and prevalence from major types of cancer for 184 countries of the world based on 2012 data. The project found that an estimated 14.1 million new cancer cases occurred around the world and this staggering number is expected to increase to 24 million by 2035. In addition, an estimation of 8.2 million cancer deaths have occurred and 32.6 million people were

living with cancer (within 5 years of diagnosis) in 2012 worldwide (Ferlay *et al*., 2014). With the increase of human life expectancy, so is the incidence of malignant tumors which increases at speed 1.5 to 2.0% every 10 years (Hunold *et al*., 2006).

In Malaysia, based on 2015 record from the Ministry of Health, cancer which contributed to 13.56% of all deaths, was the third most common cause of death after diseases of circulatory system (22.77%) and diseases of the respiratory system (18.54%), where it was also reported that during the period of 2007 to 2011, 103 507 deaths have occurred due to cancer (Azizah *et al*., 2015).

1.1.3 Hallmarks of cancer

Cancer is a complex disease which operates in multiple ways. Hallmarks of cancer include at least a few of the characteristics as depicted in Figure 1.1 (Hanahan and Weinberg, 2011).

1.1.4 Chemotherapy drugs

Many forms of chemotherapy are targeted at the process of cell division. The rationale being that cancer cells are more likely to be replicating than normal cells. Chemotherapy drugs include a number of families defined by both their chemical structures and mechanisms of action: alkylating agents, antimetabolites, cytotoxic antibiotics, topoisomerase inhibitors, spindle poisons and platinum compounds (Espinosa *et al*., 2003; Payne and Miles, 2008). Other more advanced cancer treatments include targeted

therapy, hormone therapy, immunotherapy, stem cell transplant and precision medicine (National Cancer Institute, 2017).

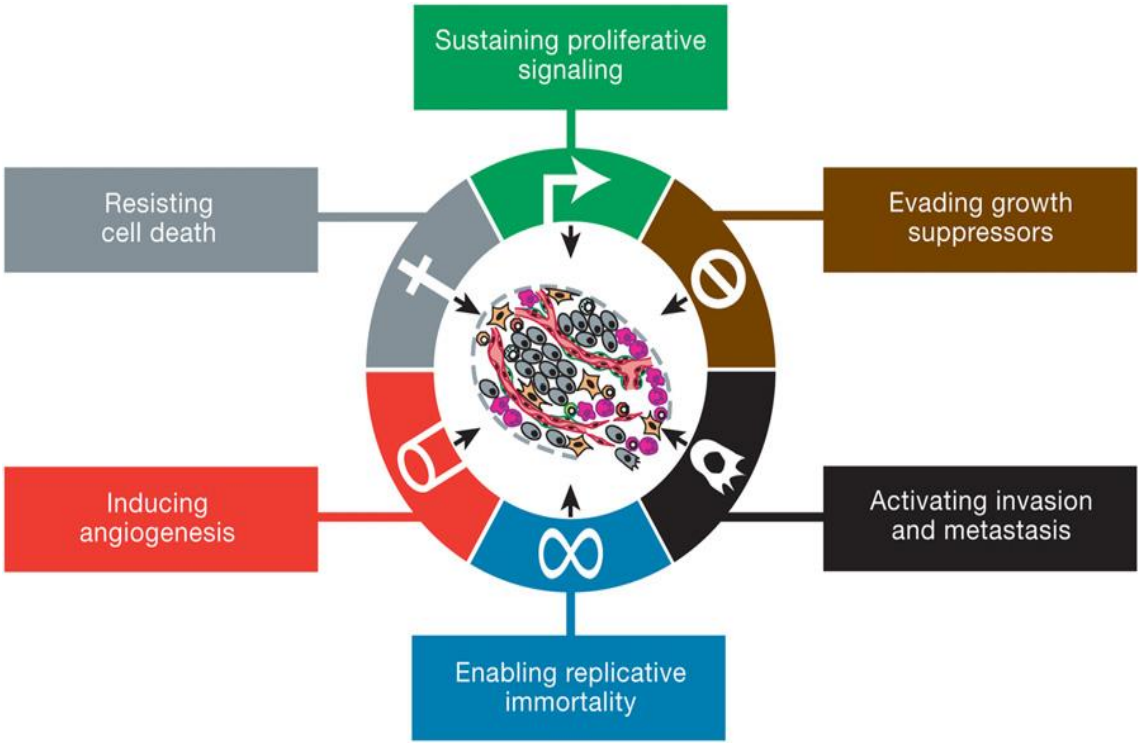

Figure 1.1. Hallmarks of cancer (Source: Hanahan and Weinberg, 2011).

At present, chemotherapeutics are the main source of treatments in the management of cancer. Even with the arrival of targeted-therapy revolution, the principles and limitations of chemotherapy discovered by the early researchers still apply till today (Chabner and Roberts, 2005). The primary reasons for treatment failure in cancer chemotherapy are the infamous severe side effects to the normal cells and the ever-increasing emergence of the multidrug resistance of cancer cells to chemotherapy (Harleva *et al*., 2012; Leite de Oliveira *et al*., 2012; Holohan *et al*., 2013).

1.1.5 Leukemia

Leukemia is a cancer of the blood, specifically the white blood cells, and is common among both adults and children (Wiseman, 2008; Siegel *et al*., 2012; Teichroew, 2016). Leukemia incidence rates have been rising on average 0.3% each year over the last 10 years, though death rates have been falling on average 1.0% each year over 2005 to 2014 (National Cancer Institute, 2016). It is one of the most common cancers worldwide, and it is also number one in causing childhood cancer, by up to 34% of all types of cancer in children (Wiseman, 2008; Siegel *et al*., 2012). Among the Malaysian population, leukemia is the sixth most common cancer in adults as well as the number one most common cancer among children (Azizah *et al*., 2015).

There are four primary types of leukemia which are categorized based on their rates of progression and where the cancer develops; acute myeloid (or myelogenous) leukemia (AML), chronic myeloid (or myelogenous) leukemia (CML), acute lymphocytic (or lymphoblastic) leukemia (ALL) and chronic lymphocytic leukemia (CLL) (American Cancer Society, 2017; Teichroew, 2016). In this study particularly, leukemia of interest is CML, which was represented by K562 cell line.

1.1.6 Chronic myelogenous leukemia (CML)

Compared to other types of leukemia, CML is most commonly diagnosed among adults with an incidence rate of 1-1.5 per 100 000 people, and represents approximately 15% of all leukemias with onset age at 40–60 years. On the other hand, children and adolescents

make up 3% of diagnosed cases (Ries et al., 1999; Jabbour and Kantarjian, 2012; American Cancer Society, 2017a). This malignant hematopoietic disorder which is characterized by uninhibited proliferation of abnormal and undifferentiated blood progenitor/stem cells (specifically myeloid cells), also known as blast cells (Druker et al., 2001; Kalidas et al., 2001), begins its attack in the blood-forming cells of the bone marrow. With time progression, the malignancy starts spreading to the blood and eventually circulating to other areas of the body. When blood and bone marrow are overflowed with these blast cells, healthy white blood cells, red blood cells, and platelets which usually occupy these sites have no space to proliferate. Many clinical side effects are expressed as a result of this anomaly, in the form of infections, anemia, ease of bleeding, pain in the bones and pain or fullness feeling below the ribs on the left side (Abou-Jawde *et al*., 2003; Moosavi *et al*., 2006; PDQ® Adult Treatment Editorial Board, 2017).

CML spreads and progresses slowly and it has three distinct phases; chronic, accelerated and blastic (Abou-Jawde *et al*., 2003; Moosavi *et al*., 2006; PDQ® Adult Treatment Editorial Board, 2017). These phases are determined based on two factors, which are how many blast cells are there in the blood and bone marrow and how severe the signs or symptoms are (PDQ® Adult Treatment Editorial Board, 2017). Chronic phase is characterized as having less than 10% of blast cells in the blood and bone marrow. Most CML patients are diagnosed in this phase, and disease outcome is generally easily manageable with outpatient therapy. Given approximately 3-8 years, disease progression goes to the next phase which is the accelerated phase, or straight to the blastic phase (Lee, 2000; Axdorph *et al*., 2002). In the accelerated phase, the blood

and bone marrow contain between 10-19% of blast cells, and early signs of symptoms start to appear. Further progression is to the blastic phase, where at this time, the blood and bone marrow contain 20% or more of blast cells. This phase is also called blast crisis, where symptoms such as tiredness, fever, and enlarged spleen begin to appear and the transformation may be associated with a markedly deteriorated prognosis (Lee, 2000; Axdorph *et al*., 2002; PDQ® Adult Treatment Editorial Board, 2017).

1.1.6(a) Pathogenesis of chronic myelogenous leukemia (CML)

CML has a distinct characteristic that makes it different from the other three leukemias. This distinction comes from CML's association with an abnormal chromosome known as the Philadelphia chromosome (Figure 1.2), which arises from the reciprocal translocation of genes between two chromosomes, whereby a piece of chromosome 9 (ABL) and a piece of chromosome 22 (BCR) break off and exchange places in pluripotent hematopoietic stem cells. Formation of this new BCR-ABL gene is on chromosome 22 where the piece from chromosome 9 attaches, and the changed chromosome 22 is now called the Philadelphia chromosome (Deininger *et al*., 2000; O'Dwyer, 2002; PDQ® Adult Treatment Editorial Board, 2017). The BCR-ABL oncogene encodes a fusion protein p210 BCR-ABL with deregulated tyrosine kinase, which is responsible for cell growth independent of growth factor, survival of myeloid progenitor cells, apoptotic resistance, arrested lymphoid development and multistep oncogenic progression, thus responsible for leukemogenesis (Deininger *et al*., 2000; O'Dwyer, 2002; Abou-Jawde *et al*., 2003). The discovery and widespread prescription of BCR-ABL tyrosine kinase inhibitors has benefited CML patients due to an increase in

the 5-year survival rate of 55.2% for diagnosed patients from 2001 to 2007, in comparison to back in 1990 to 1992, when their 5-year survival rate was only 31.0% (Siegel *et al*., 2012).

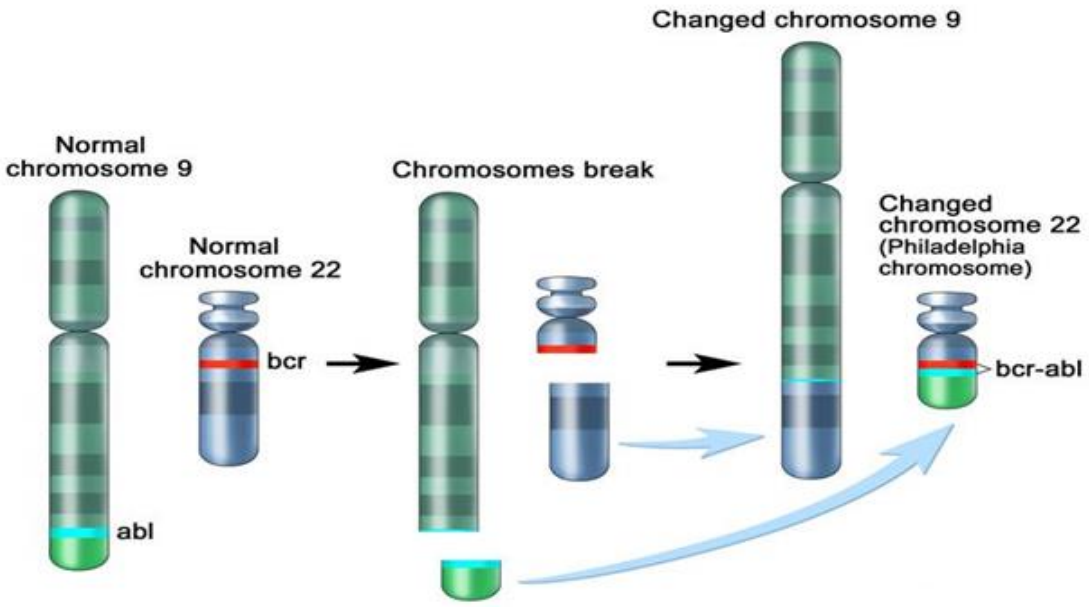

Figure 1.2. Translocation process of fusion product, BCR-ABL oncogene, also known as Philadelphia chromosome between chromosomes 9 and 22 (Source: PDQ® Adult Treatment Editorial Board, 2017).

1.1.6(b) Failed strategy to target BCR-ABL gene

The pathogenesis of CML which is derived from the formation of BCR-ABL gene constitutes an attractive target for antisense strategies. The principle of antisense strategies relies on the formation of duplex complexes between the target mRNA and exogenous antisense oligonucleotide (short DNA or RNA nucleotides), therefore preventing the action of ribosomal complex to read and translate the transcribed message (Gewirtz *et al*.,1998; Galderisi *et al*., 1999; Salesse and Verfaillie, 2002). Unfortunately, such strategies failed to deliver due to some technical problems. The long half-life of

p210 BCR-ABL (> 24 hours) required extended *ex vivo* culture to induce leukemic cell death and such would interfere with the engraftment ability of hematopoietic progenitors (Spiller *et al*., 1998; Spiller *et al*., 1998a; Clark, 2000; Salesse and Verfaillie, 2002). In addition, there has been evidence showing that the mRNA and protein of BCR-ABL gene were not expressed in CML stem cells, so treatment with antisense oligonucleotide failed to eliminate the leukemic cells (Salesse and Verfaillie, 2002). Therefore, in an effort to target the BCR-ABL gene effectively, drug inhibitors of the BCR-ABL fusion protein were developed, known as tyrosine kinase inhibitor (TKI) (Druker *et al*., 2003).

1.1.6(c) Imatinib

The most common drug for CML is imatinib, a moderately potent inhibitor of the kinase BCR–ABL, whose action is to inhibit tyrosine phosphorylation of proteins involved in BCR-ABL signal transduction by competitively inhibiting the adenosine triphosphate binding site of the tyrosine kinase enzyme. As a result of such mechanism of action, growth arrest or even apoptosis caused by imatinib occurs on BCR-ABL gene expressed hematopoietic cells without inflicting the normal cells (Druker *et al*., 2003). Patients with chronic-phase CML benefit from this drug, whereby about 80-90% would achieve complete haematological remission and many would lose cytogenetic evidence of the malignant clone (Chabner and Roberts, 2005; Radich, 2007). However, the progression of CML affects the effectiveness of imatinib therapy. For accelerated phase, the complete cytogenetic response rate reduced to 40%, and this number reduced further to only 20% for patients in the blast crisis. The reason for such profile might stem from

the fact that cells are more exposed to genomic instability as a result of longer activation of BCR-ABL prior to initiation of therapy (Radich, 2007).

1.1.6(c)(i) Problems with imatinib treatment

The use of imatinib has been shown to induce mild to moderate toxicity, though adverse effects could occur within the two years of the start of therapy. Just like any other chemotherapy drugs, imatinib comes with a few side effects such as nausea, lethargy, edema, myalgias, diarrhea and myelosuppression (Druker et al., 2003). Some patients also suffer through conditions of rashes' development, debilitating bone pain, oligospermia, gynecomastia and on some occasions, hepatotoxicity and cardiotoxicity (Grey *et al*., 2006; Seymour *et al*., 2006; Mughal and Schrieber, 2010).

In spite of the side effects, the major issue with imatinib treatment for CML is the development of resistance, whereby up till now, approximately 100 different BCR–ABL kinase domain mutations have been reported (Ernst and Hochhaus, 2012). The failure of the TKI to effectively eradicate leukemic cells could be attributed to an increase in efflux transport through the activation of various drug efflux transmembrane proteins in the forms of adenosine triphosphate (ATP)-binding-cassette (ABC) transporter family P-glycoprotein (Pgp), multidrug resistance-associated protein 1 (MRP1) and breast cancer resistance protein (BCRP) (Chen and Sikic, 2012). Other mechanisms of resistance include decrease in influx transport (White *et al*., 2006) as well as increased resistance to apoptosis due to loss or mutation of apoptotic components such as p53 and members of the B-cell lymphoma 2 **(**BCL-2) family (regulation of the

intrinsic pathway) and extrinsic death receptor pathway (Honda *et al*., 2000; Selleri and Maciejewski, 2000; Aichberger *et al*., 2005; Gonzalez *et al*., 2010).

In an effort to overcome this resistance problem, second generation of selective BCR–ABL TKIs such as nilotinib and dasatinib have been developed (Talpaz *et al*., 2006; Kantarjian *et al*., 2007). Unfortunately for a minority of CML patients in the chronic phase as well as some substantial proportion in the advanced phases, they are either initially refractory or eventually develop resistance to these drugs (Ernst and Hochhaus, 2012).

Even after treatment and supposed remission, the advanced technology of polymerase chain reaction (PCR) analysis can still detect BCR–ABL oncogene in cells of most patients (Druker *et al*., 2001; Kantarjian *et al*., 2002; Hughes *et al*., 2003). These mutational abnormalities cause the enzyme to stay in its open configuration, leading to poor binding of the drug but remaining catalytically active (Shah *et al*., 2002). Moreover, there is evidence that drug-resistant cells are present even before drug exposure for some of these patients with chronic phase CML, (Roche-Lestienne *et al*., 2002), a finding which is usually associated with rapid emergence of resistance (Branford et al., 2003). In the blast phase of CML, imatinib can only induce short remissions as the treatment introduces rapid outgrowth of cells which are mutated and drug resistant (Chabner and Roberts, 2005).

In this study, *in vitro* investigation of CML was represented through the use of K562 cell line. Other cell lines were also utilized to represent other types of cancer, namely HT-29 for colon cancer, HeLa for cervical cancer and Hep G2 for liver cancer. However, this study emphasized on K562 cell line as the β-carboline compounds have shown highest potency and selectivity to this particular cell line (sections 3.1.1 and 3.1.2), in comparison to the other three cell lines.

1.1.7 K562 cell line

K562 is an erythroblastic cell line which has been established from the pleural extravasation of a patient with CML in blast crisis and behaves as a pluripotent hematopoietic stem cell (Klein *et al*., 1976). It expresses the typical pathogenic hallmark of human CML which is the fusion protein product of a chromosomal translocation Philadelphia chromosome (BCR-ABL oncogene) (Lozzio and Lozzio, 1975; Law *et al*., 1993; Deininger *et al*., 2000; O'Dwyer, 2002; Pastwa *et al*., 2005). In addition, K562 cells also have mutated p53 gene, which is a tumor suppressor gene required for the induction of programmed cell death initiated by DNA damage (Lozzio and Lozzio, 1975; Moosavi *et al*., 2006). This cell line has been extensively used as a suitable and worldwide model for *in vitro* investigation of CML and to probe into the metabolic pathways underlying its therapy (Law *et al*., 1993). K562 cell line has been also utilized in *in vitro* experimental system for the study of differentiation potential of many compounds (Klein *et al*., 1976; Law *et al*., 1993; Clarkson *et al*., 2003). Perhaps due to the combination of the p53 mutation and BCR-ABL oncogene expression, the K562 cell line displayed a relatively high level of resistance to most cytotoxic drugs, hence it has

been the object of experiments to test resistance in apoptosis-inducing drugs (Martin *et al*., 1990; McGahon *et al*., 1994; Dubrez *et al*., 1998).

1.1.8 β-carboline

β-carboline can be derived from a large group of naturally occurring and synthetic alkaloids. The general scaffold is characterized by a planar tricyclic system, which consists of pyridine ring that is fused to an indole skeleton, as shown in Figure 1.3 (Love, 2006; Cao *et al*., 2007).

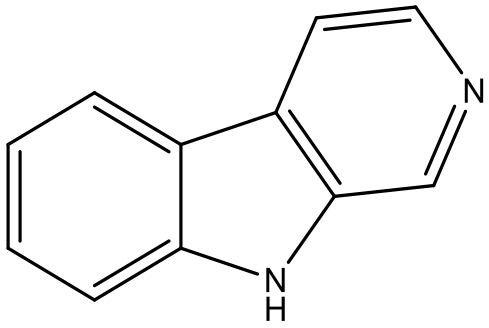

Figure 1.3. General scaffold of β-carboline depicting a planar tricyclic system.

1.1.8(a) Origins

β-carboline is structurally related to harmine, an active alkaloid originally isolated from a plant called *Peganum harmala* L. family Zygophyllaceae (also known as harmal, harmel, Syrian rue, espand, etc.) (Kuo *et al*., 2003; Herraiz *et al*., 2010). Other major active components of *Peganum harmala*'s extracts are identified to be harmol, harmaline, harmalol and harman, which are all β-carboline alkaloids (Lamchouri *et al*.

1999; Herraiz *et al*., 2010). *Peganum harmala* is a perennial herbaceous plant native and widely used as a medicinal plant in Iran, Central Asia, North Africa, Middle East, Mediterranean Sea, Pakistan and India, where it was later introduced and naturalized in parts of the southwest USA, and a few areas of South Africa and Australia (Sheahan and Chase, 2000; Frison *et al*., 2009; El Gendy and El-Kadi, 2009; Herraiz *et al*., 2010; Wanntorp and Ronse De Craene, 2011).

1.1.8(b) Traditional uses

Since ancient times, β-carboline has been known for a plethora of traditional uses. Some of the treatments of β-carboline are for hypertension, cardiac diseases, diabetes (Tahraoui *et al*., 2007; Bnouham *et al*., 2002), depression (Brobst *et al*., 2009), antipyretic (Farouk *et al*., 2008), antispasmodic, emetic (Prashanth and John, 1999), hallucinogenic, antibacterial (Sokmen *et al*., 1999), antimalarial (Chen *et al*., 2004), pain relief, diarrhea, intestinal pain, asthma (Farouk *et al*., 2008), alimentary tract cancers (Chen *et al*., 2004), emmenagogue and abortifacient (Monsef *et al*., 2004). More known traditional uses of β-carboline are listed in a review by Moloudizargari *et al*. (2013).

1.1.8(c) Pharmacological effects

β-carboline has a wide spectrum of action especially on muscular, cardiovascular and central nervous systems (Aqel and Hadidi, 1991; Farouk *et al*., 2008; Moura *et al*., 2007). Hence its pharmacological effects are even more varied, as the ring system from β-carboline scaffold has been shown to be effective for many biological activities;

anticancer (Plassmann *et al*., 2005), antioxidant (Moura *et al*., 2007), antiinflammatory (Trudell *et al*., 1992), antidepressant (Castro *et al*., 2003), anticonvulsant, antidiabetic (Fortunato *et al*., 2009), antibacterial (Nenaah, 2010), antiviral, antiparasitic (Alomar *et al*., 2013; Cao *et al*., 2007), antiarrhythmic, antimicrobial (Saadabi, 2006; Savariz *et al*., 2012), antispasmodic, anticholinergic (Aqel and Hadidi, 1991), neuroprotection (Maher and Davis, 1996), analgesic (Farouk *et al*., 2008), sedative, anxiolytic (Schlecker *et al*., 1995; Batch and Dodd, 1998) and treatment for erectile dysfunction (Ahmed *et al*., 2012). More known pharmacological effects of β-carboline are listed in a review by Moloudizargari *et al*. (2013).

1.1.8(d) Adverse effects

Though not as widely reported as the pharmacological effects, there are some reports of the detrimental effects of β-carboline. There is strong evidence that β-carboline alkaloid is highly neurotoxic and causes tremor in a wide variety of animals (Du *et al*., 1997; Louis, 2008). This may also lead to the pathogenesis of Parkinson's disease (Polanski *et al*., 2010), as evidenced by significantly high plasma levels of norharmane and harmane (alkaloids of β-carboline) in Parkinsonian patients compared to the control group (Kuhn et al., 1995). Overdose ingestion of *Peganum harmala* for medicinal use or as a recreational psychoactive product can be poison and several cases of toxicity have been already reported in animals and humans as it has been found to produce paralysis, euphoria, convulsions, hallucinations, digestive problems (nausea, vomiting), hypothermia and bradycardia (Ben Salah *et al*., 1986; Elbahri and Chemli, 1991; Frison *et al*., 2009; Mahmoudian *et al*., 2002).

1.1.8(e) Anticancer activity of β-carboline

The emergence of anticancer potential of β-carboline has attracted a lot of scientific attention. Some of the β-carboline extracts have shown significant tumor inhibition effect in tumor-bearing mice and *in vitro* studies have also shown that β-carbolines, such as harmine, are highly cytotoxic and significantly inhibit tumor cell growth through apoptosis (Ishida *et al*., 1999; Lamchouri *et al*., 1999; Uezono *et al*., 2001; Kuo *et al*., 2003). In fact, some papers have reported that β-carboline elicited even better anticancer activities in a wide range of cancer cell lines in comparison to known anticancer drugs cisplatin, carboplatin and 5-fluorouracil (5-FU) (Al-Allaf and Rashan, 1998; Yoshino *et al*., 1999; Cao *et al*., 2013). *In vivo* studies using mice bearing cancer cells also showed better antitumor activity compared to anticancer drug cyclophosphamide (Cao *et al*., 2013). Moreover, there are even more anticancer studies of β-carboline which have shown good killing activity against a broad range of human cancer cell lines such as K562, HT-29, HeLa, Hep G2, U251, MCF-7, UACC-62, PCO-3, OVCAR-03, NCI-460, Bel-7402, BGC-823, A549, SCaBER, Blu-87 and KB (Cao *et al*., 2004; Cao *et al*., 2005; Shen *et al*., 2005; Formagio *et al*., 2008; Han *et al*., 2012; Savariz *et al*., 2012).

1.1.8(f) Mechanisms of action of β-carboline

Literature has reported that β-carboline compounds exhibit their anticancer activity through multiple mechanisms. They mediate their action mainly through DNA intercalation as well as inhibiting cyclin-dependent kinases (CDKs) and DNA topoisomerases (Hayashi *et al*., 1977; Taira *et al*. 1997; Csányi *et al*., 2000; Deveau *et*

al., 2001; Xiao *et al*., 2001; Song *et al*., 2002; Nii, 2003; Song *et al*., 2004). Other reported targets include cytochrome P450, monoamine oxidase A, mitogen activated protein kinase-activated protein kinase 2 (MK-2), pololike kinase-1 (PLK1), IκK (IκB kinase complex), kinesin Eg5 and dual enzyme tyrosine phosphorylated and regulated kinase 1a (DYRK1A) as well as benzodiazepine, serotonin, dopamine and imidazoline receptors (Fernandez de Arriba *et al*., 1994; Pimpinella and Palmery, 1995; Funayama *e*t *al*., 1996; Kim *et al*., 1997; Grella *et al*., 1998; Stawowy *et al*., 1999; Glennon *et al*., 2000; Husbands *et al*., 2001; Castro *et al*., 2003; Squires *et al*., 2004; Trujillo *et al*., 2007; Zhang *et al*., 2009; Barsanti *et al*., 2010; Herraiz *et al*., 2010; Yadav and Nandi, 2014). The apparent and wide array of differences in mechanisms of action may be attributed to the differential structures as well as the target of cancer cells.

1.1.9 Evaluating potential anticancer activity of β-carboline

All these reports have piqued the current interest in investigating anticancer effects of β-carboline, and justified the purpose of the present study. Brief introductions on the methods undertaken in this study are mentioned in the following sections.

1.1.9(a) Cytotoxic activity using 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay

In this study, MTT assay was used to evaluate cell viability in cancer cells as a primary and secondary screenings, and it has also been adapted differently to investigate other parameters. MTT assay evaluates viable cells through the estimation of their metabolic

activity, and its widely use has been well recognized worldwide. In principle, the viable cells contain mitochondrial dehydrogenase, which acts to cleave yellow dye of MTT and as a result of that, measurable purple formazan is formed. Since the purple formazan is impermeable to cell membranes, it ends up accumulating in viable cells. This formazan production is a good measurement for evaluating cell growth as it is directly proportional to cell viability and inversely proportional to cytotoxicity (Butler, 2004).

The use of MTT has become the gold standard in academic labs as evidenced by thousands of published articles, not just for its versatility, but also because it has adequate amount of simplicity, accuracy and reliability as a method to count metabolically active cells (Mossmann, 1983; Denizot and Lang, 1986; Freimoser *et al*., 1999; Aziz, 2006; Riss *et al*., 2016). In addition, MTT poses several advantages over other assays as it is cost- and time-saving, works equally well for adherent and suspension cells and adaptable for high throughput of larger number of compounds/samples, which makes it suitable for primary screening (Pieters *et al*., 1988; Freimoser *et al*., 1999; Weyermann *et al*., 2005; Aziz, 2006). Moreover, the National Cancer Institute (NCI) has recommended the use of MTT assay as part of its *in vitro* anticancer drug discovery screen (Boyd, 1997). MTT assay has also been used in numerous cancer studies which utilized anticancer drugs to evaluate cell growth inhibitory effect (Istiaji *et al*., 2010; Lüpertz *et al*., 2010; Sharifi *et al*., 2015). In fact, based on the recommendations from an international workshop organized by the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), National Toxicology Program (NTP) and Interagency Center for the Evaluation of Alternative Toxicological Methods (ICEATM), MTT assay was one of the

listed methods used for measurement endpoints for basal cytotoxicity (National Toxicology Program, 2001). Thus, MTT seemed to be the best selection to study viability of cells in this study.

1.1.9(b) Acridine orange and ethidium bromide (AO/EB) fluorescence assay

Apoptosis, also known as programmed cell death, is a well-controlled and tightly-regulated physiological process which results in self-destruction of cells (Baskic´ *et al*., 2006). It is a necessity for the normal development, homeostasis and function of the immune system as well as a response to cellular insults and oncogenesis (Golstein *et al*., 1991; Cohen *et al*., 1992; Pradelli *et al*., 2010). Apoptosis is characterized by a sequence of specific morphological changes in preparation for cell death. It begins with the condensation of the cytoplasm and nuclear chromatin, followed by membrane blebbing and breakage of cells into apoptotic bodies, which still contain various cytoplasmic organelles and nuclear fragments. Next, phagocytic cells and macrophages which are nearby act to rapidly engulf these apoptotic bodies, and finally digest them in their lysosomes (Golstein, 1998; Van Herreweghe *et al*., 2010). The clearance system is so effective that little cell death is apparent (Golstein, 1998).

On the other hand, there is another form of unfavorable cell death called as necrosis, and also termed as accidental cell death. Known as the alternative and toxic version to apoptotic cell death, necrosis causes cell to become a passive victim of cell death in a mode of death that is independent of energy. The process of necrosis is degradative, which leads to mitochondria swelling and rupture of plasma membrane. As

a result of that, inflammatory cellular contents are released to the surrounding area of the cell, leading to the presentation of self-antigens in an immunogenic form and ultimately causing tissue damage due to autoimmunity (Majno and Joris, 1995; Levin *et al*., 1999; Nagata *et al*., 2010).

The distinction between these two types of cell death is essential to recognize the possible physiological or pathological effects which are caused by apoptosis and necrosis respectively. Hence reliable methods for detecting cell death and differentiating between apoptosis and necrosis are most vital. In this study, microscopic examination of AO/EB fluorescence staining of cells was used as it was deemed most suitable since it enables the performance of high-quality studies of cell morphology, nuclear and chromatin disintegration. Though the use of dyes AO and EB, this assay is capable of distinguishing viable and non-viable cells based on membrane integrity, giving this assay the ability to distinguish viable, early or late apoptotic and necrotic cells in a rapid and objective manner (Ashush *et al*., 2000; Baskic´ *et al*., 2006).

1.1.9(c) Measuring the levels of caspase-3 and -7

The intracellular machinery responsible for apoptosis seems to be similar in all mammalian cells. This machinery depends on an evolutionary conserved family of proteases that have a cysteine at their active site and cleave their target proteins at specific aspartic acids, and they are called caspases (Liu *et al*., 2005). Caspases play essential roles as apoptosis initiator and executor of mammalian cells (Lamkanfi *et al*.,

2002). Apoptosis-inducing stimuli such as chemotherapy drugs induce apoptosis through the caspase-mediated pathway (Hensley *et al*., 2013).

Known as the heart of the apoptotic machinery, caspases typically signal in a two-step cascade, beginning with recruitment of 'initiator caspases' with large pro-domains (such as caspase-1, -8, -9, -10) forming into large protein complexes in which they undergo proximity-induced autoactivation. From here, they act to specifically free 'executioner caspases' (caspase-3, -6, -7) of their short inhibitory prodomain and allowing them to cleave a large set of cellular substrates (Stennicke *et al*., 1998; Slee *et al*., 1999; Boatright *et al*., 2003). This amplifying proteolytic cascade of caspase activation is followed by degradation of cellular components, resulting in caspase-dependent apoptosis (Rathmell and Thompson, 1999; Igney and Krammer, 2002; Degterev and Yuan, 2008).

There are two classical pathways of apoptosis, which are the intrinsic mitochondrial pathway and the extrinsic death receptor pathway. Looking at Figure 1.4, the intrinsic pathway is regulated by B-cell lymphoma 2 (BCL-2) family members while the extrinsic pathway is regulated by FAS- and tumour necrosis factor receptor (TNFR)-associated death domains. While each pathway is operated by multiple activation and inhibition of different types of proteins, both pathways eventually converge at the activation of the effector caspases (caspase 3, caspase 7 and caspase 6) to induce apoptosis (Li-Weber, 2013; Czabotar *et al*., 2014). Once activated, these executioner caspases are responsible for the proteolytic cleavage of a broad spectrum of cellular targets, leading ultimately to the disassembly of the cell (Liu *et al*. 2005; Li-Weber,

2013). Since caspase-3 and -7 are known to be major executioner caspases and protein markers for apoptosis, their upregulations were evaluated in this study to understand the underlying pathway of apoptosis (Walsh *et al*., 2008).

To assess the levels of caspase-3 and -7, Caspase-Glo® 3/7 of Promega was utilized. Caspase-Glo® 3/7 assay is a homogeneous, luminescent assay that provides a luminogenic caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD, in a reagent optimized for caspase activity, luciferase activity and cell lysis. Following the action of cleavage by caspase-3 and -7 as depicted in Figure 1.5, a substrate for thermostable luciferase (aminoluciferin) (Ultra-Glo™ Recombinant Luciferase) is released, resulting in the luciferase reaction and generating light or a stable "glow-type" luminescent signal, thus improving performance across a wide range of assay conditions (Promega, 2015). This assay is highly advantageous as it is a simple and robust assay which is able to provide rapid results that are highly selective for caspase-3 and -7 Bayascas *et al*., 2002; Promega, 2015). In addition, this flexible assay has a greater sensitivity than other fluorescence-based assays because it avoids interference from fluorescence signals from test compounds, providing excellent signal-to-noise ratios and allowing the use of fewer cells in the study of apoptosis. This assay also enables the use of cultures of adherent or suspension cells, and its multiwell-plate format makes it ideal for automated high-throughput screening of caspase activity or apoptosis (Promega, 2015).

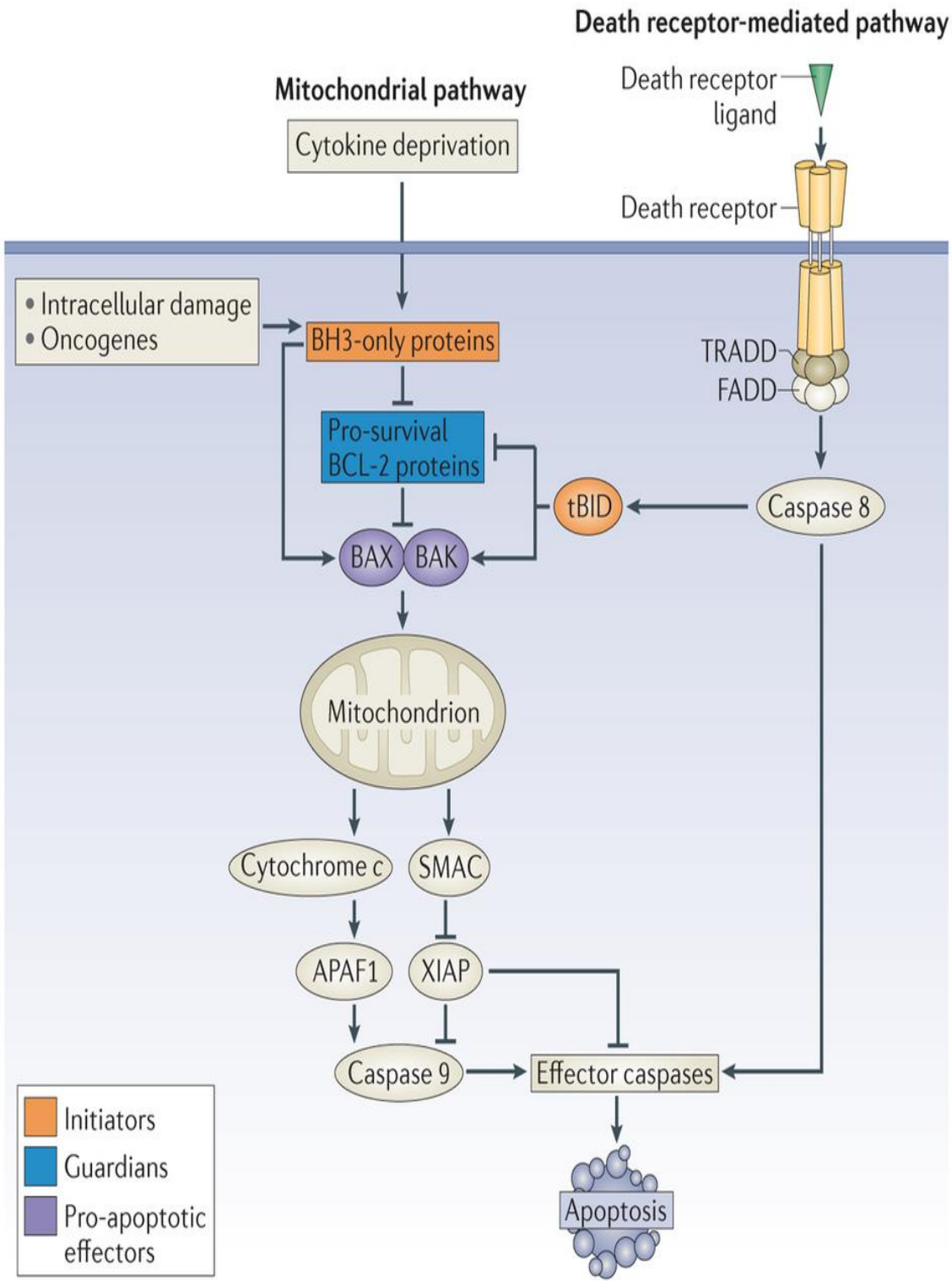

Figure 1.4. The intrinsic mitochondrial- and extrinsic death receptor-mediated pathways to apoptosis (Source: Czabotar *et al*., 2014).

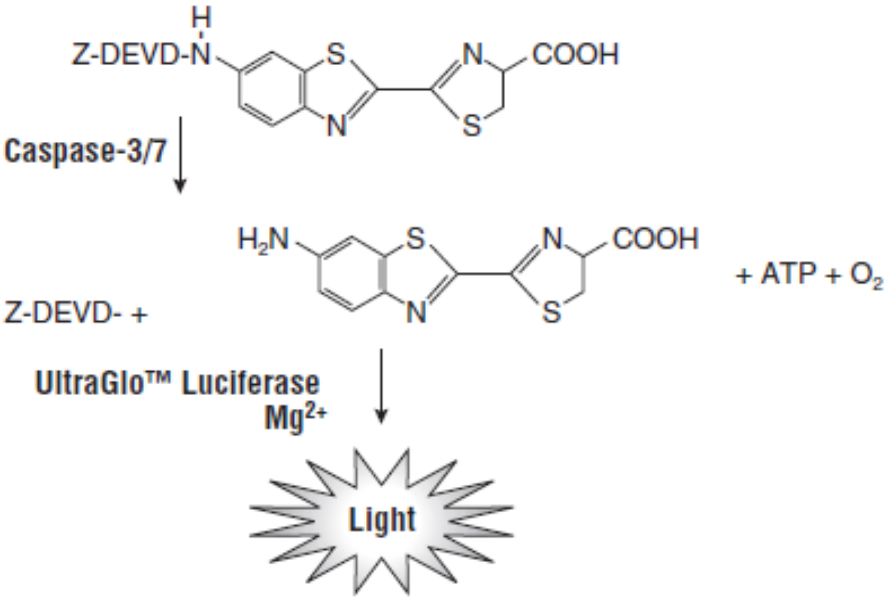

Figure 1.5. Caspase-3/7 cleavage of the luminogenic substrate containing the DEVD sequence, thereby releasing a substrate for luciferase (aminoluciferin) and resulting in the luciferase reaction and the production of light (Source: Promega, 2015).

1.1.9(d) Flow cytometry

The stages of the cell cycle are divided into two major phases which are interphase (consist of G_1, S, G_2) and mitotic (M) phase (Figure 1.6). During interphase, cell growth and DNA duplication occur, while during the M phase, cell separates its DNA into two sets, followed by division of its cytoplasm, and forming two new daughter cells. Different stages of the cell cycle are characterized by the different amounts of DNA that the cell nuclei contain. During the G_1 phase, cells maintain diploidy (DNA content characteristic of two complete sets of chromosomes, 2N) as they undergo RNA and protein synthesis to prepare for DNA synthesis. Subsequently, DNA synthesis and replication occur in the S phase, where DNA contents are intermediary between G_1 and G_2 (2N to 4N DNA content). During the G_2 phase, in preparation for the M phase, more

RNA and protein synthesis occur, and therefore DNA content is doubled and defined as G_2 tetraploid (4N DNA content). At this phase, based on DNA content alone, M phase is indistinguishable from the G_2 phase. In the process of mitosis, after the split of cell into two 2N daughter cells, these new cells either continue to enter another cycle or go into a resting phase defined as G_0. Based on DNA content, this phase is indistinguishable from G_1 phase. Collectively, the replication cycle in flow cytometry is described by G_0/G_1, S, and G_2/M phases (Wang *et al*., 2011).

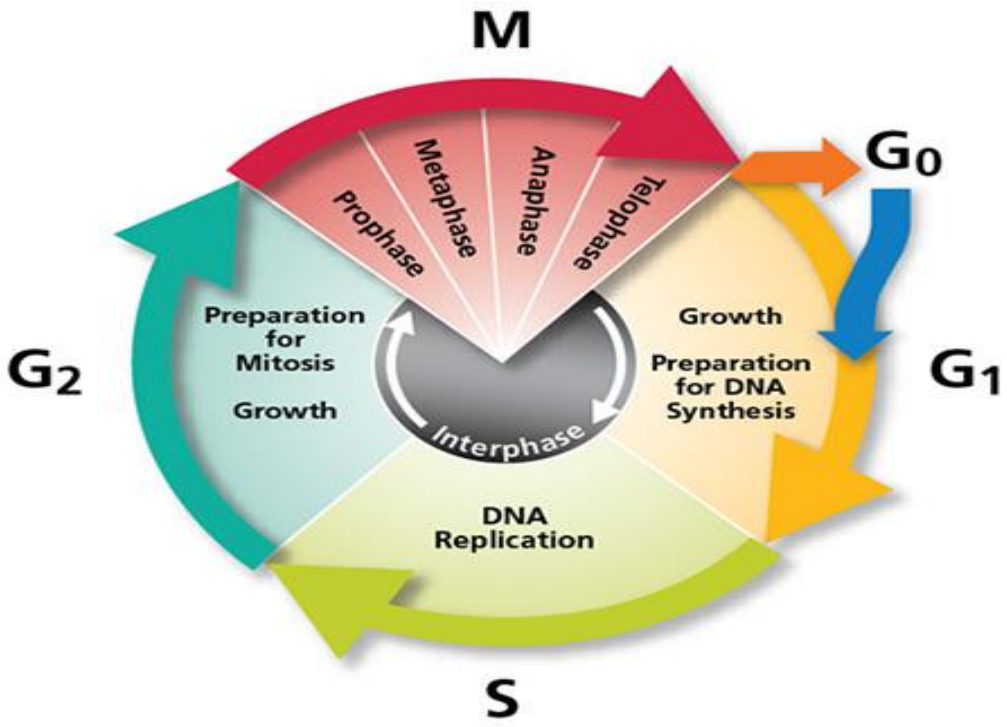

Figure 1.6. The stages of cell cycle (Source: Wang *et al*., 2017).

Flow cytometry can measure DNA content of cells, which reveals not only the information on cell position in the cell cycle but also the ploidy and DNA content of a given cell population (Pozarowski and Darzynkiewicz, 2004; Wang *et al*., 2011).